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(54) Title: STRUCTURAL GENE OF PNEUMOCOCCAL PROTEIN

(57) Abstract

A purified pneumococcal surface protein A (PspA) comprises a truncated form of the PspA protein which is immunoprotective and contains the protective epitopes of PspA. The PspA protein is soluble in physiologic solution and lacks at least the cell membrane anchor region of the whole protein. The protein is formed by insertion-duplication of mutagenesis of *S. pneumoniae* with *pspA* gene and expression of the truncated protein into the growth medium.

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TITLE OF INVENTION

STRUCTURAL GENE OF PNEUMOCOCCAL PROTEIN

FIELD OF INVENTION

The present invention is concerned with the development of an improved vaccine against pneumococcal infections.

REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of copending United States patent application Serial No. 656,773 filed February 15, 1991.

BACKGROUND TO THE INVENTION

Streptococcus pneumoniae is an important cause of otitis media, meningitis, bacteremia and pneumonia. Despite the use of antibiotics and vaccines, the prevalence of pneumococcal infections has declined little over the last twenty-five years.

It is generally accepted that immunity to Streptococcus pneumoniae can be mediated by specific antibodies against the polysaccharide capsule of the pneumococcus. However, neonates and young children fail to make an immune response against polysaccharide antigens and can have repeated infections involving the same capsular serotype.

One approach to immunizing infants against a number of encapsulated bacteria is to conjugate the capsular polysaccharide antigens to protein to make them immunogenic. This approach has been successful, for example, with Haemophilus influenzae b (see U.S. Patent no. 4,496,538 to Gordon and U.S. Patent no. 4,673,574 to Anderson). However, there are over eighty known capsular serotypes of S. pneumoniae of which twenty-three account for most of the disease. For a pneumococcal polysaccharide-protein conjugate to be successful, the capsular types responsible for most pneumococcal infections would have to be made adequately immunogenic. This approach may be difficult, because

the twenty-three polysaccharides included in the presently-available vaccine are not all adequately immunogenic, even in adults.

An alternative approach for protecting children, and also the elderly, from pneumococcal infection would be to identify protein antigens that could elicit protective immune responses. Such proteins may serve as a vaccine by themselves, may be used in conjunction with successful polysaccharide-protein conjugates, or as carriers for polysaccharides.

In McDaniel et al (I), J.Exp.Med. 160:386-397, 1984, there is described the production of hybridoma antibodies that recognize cell surface polypeptide(s) on S. pneumoniae and protection of mice from infection with certain strains of encapsulated pneumococci by such antibodies. This surface protein antigen has been termed "pneumococcal surface protein A" or PspA for short.

In McDaniel et al (II), Microbial Pathogenesis 1:519-531, 1986, there are described studies on the characterization of the PspA. Considerable diversity in the PspA molecule in different strains was found, as were differences in the epitopes recognized by different antibodies.

In McDaniel et al (III), J.Exp.Med. 165:381-394, 1987, there is disclosed that immunization of X-linked immunodeficient (XID) mice with non-encapsulated pneumococci expressing PspA, but not isogenic pneumococci lacking PspA, protects mice from subsequent fatal infection with pneumococci.

In McDaniel et al (IV), Infect. Immun., 59:222-228, 1991, there is described immunization of mice with a recombinant full length fragment of PspA that is able to elicit protection against pneumococcal strains of capsular types 6A and 3.

In Crain et al, Infect.Immun., 56:3293-3299, 1990, there is described a rabbit antiserum that detects PspA

in 100% (n = 95) of clinical and laboratory isolates of strains of S. pneumoniae. When reacted with seven monoclonal antibodies to PspA, fifty-seven S. pneumoniae isolates exhibited thirty-one different patterns of reactivity.

The PspA protein type is independent of capsular type. It would seem that genetic mutation or exchange in the environment has allowed for the development of a large pool of strains which are highly diverse with respect to capsule, PspA, and possibly other molecules with variable structures. Variability of PspA's from different strains also is evident in their molecular weights, which range from 67 to 99 kD. The observed differences are stably inherited and are not the result of protein degradation.

Immunization with a partially purified PspA from a recombinant λ gt11 clone, elicited protection against challenge with several S. pneumoniae strains representing different capsular and PspA types, as described in McDaniel et al (IV), Infect. Immun. 59:222-228, 1991. Although clones expressing PspA were constructed according to that paper, the product was insoluble and isolation from cell fragments following lysis was not possible.

While the protein is variable in structure between different pneumococcal strains, numerous cross-reactions exist between all PspA's, suggesting that sufficient common epitopes may be present to allow a single PspA or at least a small number of PspA's to elicit protection against a large number of S. pneumoniae strains.

In addition to the published literature specifically referred to above, the inventors, in conjunction with co-workers, have published further details concerning PspA's, as follows:

1. Abstracts of 89th Annual Meeting of the

American Society for Microbiology, p. 125, item D-257, May 1989;

2. Abstracts of 90th Annual Meeting of the American Society for Microbiology, p. 98, item D-106, May 1990;
3. Abstracts of 3rd International ASM Conference on Streptococcal Genetics, p. 11, item 12, June 1990;
4. Talkington et al, Infect. Immun. 59:1285-1289, 1991;
5. Yother et al, J. Bacteriol. 174:601-609, 1992; and
6. Yother et al, J. Bacteriol. 174:610-618, 1992.

The latter three publications occurred after the filing of the aforesaid USSN 656,773.

In the specification which follows and the drawings accompanying the same, there are utilized certain accepted abbreviations with respect to the amino acids represented thereby. The following Table I identifies those abbreviations:

TABLE I

AMINO ACID ABBREVIATIONS

A = Ala = Alanine	M = Met = Methionine
C = Cys = Cysteine	N = Asn = Asparagine
D = Asp = Aspartic Acid	P = Pro = Proline
E = Glu = Glutamic Acid	Q = Gln = Glutamine
F = Phe = Phenylalanine	R = Arg = Arginine
G = Gly = Glycine	S = Ser = Serine
H = His = Histidine	T = Thr = Threonine
I = Ile = Isoleucine	V = Val = Valine
K = Lys = Lysine	W = Try = Tryptophan
L = Leu = Leucine	Y = Tyr = Tyrosine

SUMMARY OF INVENTION

The present invention relates to the preparation of mutants of S. pneumoniae that secrete an immunogenic truncated form of the PspA protein, and isolation and

purification of the secreted protein. The truncated form of the PspA protein is immunoprotective and contains the protective epitopes of PspA. The PspA protein is soluble in physiologic solution and lacking at least the functional cell membrane anchor region.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 is a schematic representation of the domains of the mature PspA;

Figure 2 is the N-terminal amino acid sequence of PspA, wherein bold upper case letters denote charged hydrophilic amino acids, lower-case letters designate apolar, hydrophobic residues, and underlined bold lower case letters denote uncharged, polar, hydrophilic residues;

Figure 3 is the DNA sequence of the pspA gene with deduced amino acid sequence for the PspA protein;

Figure 4 depicts the restriction map of pspA (Figure 4A) and the use of insertion-duplication mutagenesis to construct mutations in the pspA gene (Figure 4B),

Figure 5 shows the deduced amino acid sequence for the N-terminal region of PspA and the general location of epitopes recognized by monoclonal antibodies;

Figure 6 shows antibody reactivity with PspA fragments produced by various pspA gene segments; and

Figure 7 shows the mapped location of epitopes in the PspA fragments produced by the different pspA gene segments.

GENERAL DESCRIPTION OF INVENTION

According to one aspect of the present invention, there is provided a purified immunoprotective pneumococcal surface protein, comprising a truncated form of PspA which contains the immunoprotective epitopes of the protein and up to about 90% of the whole PspA protein and from which the functional cell membrane anchor region is absent.

Through the technique of insertion-duplication mutagenesis of the pspA gene of the strain Rx1 of Streptococcus pneumoniae with plasmids containing cloned fragments of the pspA structural gene, it has been possible to produce soluble fragments of PspA that are secreted by pneumococci.

In another aspect of the present invention, therefore, there is provided a method of forming an immunoprotective truncated PspA protein, which comprises effecting insertion-duplication mutagenesis of a bacterium with a pspA gene resulting in the coding of a truncated expressible PspA protein, growing the mutated bacterium to effect expression of a truncated PspA protein, and isolating the protein.

The molecular size of the purified truncated PspA protein obtained may be varied by directing the point of insertion, which determines the termination of gene expression, to different points in the pspA gene. For example, an N-terminal fragment of apparent molecular weight of 43 kD, constituting approximately one-half of the native protein, has been found useful.

The truncated segment which is produced by this procedure is capable of eliciting protection in mice from fatal challenge with type 3 S. pneumoniae, demonstrating for the first time that a purified PspA can elicit protection and that this truncated segment of the protein contains protective epitopes of PspA.

Amino acid sequence information was obtained on the N-terminal 45 amino acids of the truncated segment of PspA. This sequence is shown in Figure 2. Predictive secondary structural analysis shows that this sequence has a very strong alpha-helical formation, with no non-helical inserts. About 51% of the segment is composed only of two amino acids, namely lysine, a charged amino acid, and alanine, a non-polar amino acid.

Analysis of this 45-amino acid sequence also reveals that it contains a seven-residue periodicity (see Figure 2). In PspA, the periodicity begins with residue 8 and extends throughout the entire sequence, for nearly eleven turns of the helix. Positions "a" and "d" are occupied by apolar amino acids and position "b", "c" and "f" generally contain hydrophilic amino acids. Position "f" is predominantly occupied by lysine. Having regard to these observations, this region of PspA is very likely in an alpha-helical coiled-coil configuration. The deduced amino acid sequence for the whole of the α-helical coiled-coil region is shown in Figure 5.

We also have cloned and sequenced the entire coding region of pspA (see Figure 3). The deduced amino acid sequence for the PspA protein reveals three distinct regions of the PspA molecule, shown schematically in Figure 1. Accordingly, a further aspect of the present invention, there is provided a biologically-pure recombinant DNA molecule coding for the PspA protein or portions thereof and having a coding sequence included within set forth in Figure 3 or having substantial homology thereto.

The DNA sequence of the pspA gene is contained on a HindIII - KpnI fragment that is 2086 base pairs in length. The pspA gene itself represents approximately 1985 base pairs of this fragment, and comprises an initial region containing transcription and translational signals with translation starting at the ATG/met (nucleotide position 127, codon position -31), followed by a leader sequence extending from the AAG/met (nucleotide position 127, codon position -31) to CGA/ala (nucleotide position 217, codon -1). Mature Pspa starts with the glu amino acid at nucleotide position 220 (codon +1) and ends at the translational stop TAA/OCH at

nucleotide position 1984. This translational stop codon is followed by transcription termination signals.

The amino terminal of the protein sequence, predicted from the DNA sequence of Figure 3, contains a 31 amino acid leader sequence and a 45 amino acid sequence identical to the 45 amino acid sequence of the N-terminal of PspA (Figure 2). The amino end of the predicted protein sequence is highly charged and α-helical in nature. This region has homology with tropomyosin at the amino acid level (approximately 22% identity and 50% similarity). This homology is due largely to a repeating seven residue periodicity where the first and fourth amino acids are hydrophobic, the intervening amino acids are helix-promoting and the seventh amino acid is charged. This pattern is consistent with that of an α-helical coiled-coil molecule and indicates that the α-helical coil extends through the N-terminal half of the molecule. The amino acid sequence of the whole of the α-helical coil region is shown in Figure 5.

Following the charged helical region is a proline-rich region in which 23 of 81 amino acids are prolines. Immediately carboxy to the proline-rich region is the first of ten highly homologous twenty amino acid repeats. The only significantly hydrophobic region in the sequenced portion of the molecule begins at the last repeat. This potential membrane-spanning region contains several charged amino acids preceding the translational stop codon.

The insertionally-inactivated mutants of S.pneumoniae lacking the C-terminal anchor regions are capable of growth in chemically-defined medium and secrete the N-terminal portion of the PspA protein into the medium. The N-terminal region of PspA is highly soluble in the culture medium and is much easier to isolate than the entire molecule. Soluble truncated

molecules have been produced using insertional duplicational mutagenesis directed by the cloned PspA DNA fragments shown in Figure 4. Expression of the same truncated construct (with the pneumococcal promoter) in E.coli results in the same PspA fragment being secreted into the periplasm of E.coli. PspA is readily released from the periplasm by hypotonic lysis.

Truncated PspA is isolated from culture medium of mutant pneumococci in any convenient manner, such as by tangential flow filtration. Ion-exchange chromatography then is performed on an anionic resin to purify the protein. In this procedure, the solution containing PspA is dialyzed to pH6 in 0.02 M salt solution and passed over the resin. The PspA is eluted from the resin with a gradient of 0.08 to 2.0 M ionic strength and is collected in the fraction between 0.34 and 0.87 M ionic strength, depending on the nature of the column used.

The PspA may be further purified by sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis. The PspA-containing portion of the gel is identified by staining of the gel and PspA is electroeluted from this portion.

The electrophoresis purification is convenient when only small quantities of PspA are being handled. As an alternative, more suited to large-scale production, the protein may be purified by size chromatography in a pH7 phosphate buffer.

Since it is possible to obtain expression of the truncated form of PspA into the culture medium, as opposed to it being trapped within the cell wall and making purification much more complicated, it is possible to isolate other proteins that have been cloned into the truncated pspA gene by making fusion proteins between PspA and other proteins. Such a technique may be employed to enhance the immunogenicity or preserve

the immunogenic structural conformation or presentation of the gene product, to permit the fusion protein to be used in immunization, which may be systemic and/or mucosal, against disease.

One example of such a fusion protein is a fusion of the soluble N-terminal region of PspA and the B-subunit of cholera toxin. Fusion proteins also may be formed by chemical attachment of the truncated PspA protein to other proteins.

Another aspect of the present invention, therefore, provides a method for the production of cloned proteins, which comprises fusing a pspA gene coding for a truncated form of PspA protein with the gene coding for another protein to form a fusion protein clone, transforming S.pneumoniae, E.coli or other bacteria with the fusion protein clone, growing the transformed bacterium to effect expression of a fusion protein comprising truncated PspA and the other protein into the culture medium, and isolating the fusion protein.

By using this technique, there can be produced cloned proteins in gram positive bacteria, such as pneumococci, for example, S.pneumoniae, and mycobacteria, for example, Bacille Calmette-Guerin (BCG). This approach overcomes the problems inherent in the production of proteins in gram negative bacteria, such as E. coli, usually used for cloning, in particular the need to purify the recombinant proteins from endotoxin and the toxicity of many gram positive DNA sequences in gram negative organisms.

For the expression of a fusion protein comprising the soluble N-terminal region of PspA and the B-subunit of cholera toxin (CTB), a gene fusion of a pspA gene coding for a truncated form of PspA protein with a ctxB gene coding for the B-subunit of cholera toxin is effected. Following expression of the fusion protein, the PspA and CTB may be cleaved one from another by

dilute acid at an asparagine-proline sequence, known to be labile to dilute acid, engineered at the fusion site of the two proteins.

CTB is known to be highly specific for monosinloganglioside (G_{M1}). Accordingly, the fusion PspA-CTB protein may be isolated from the culture medium by adsorption to a G_{M1} affinity column, from which the fusion protein subsequently may be eluted at low pH.

The PspA-CTB fusion protein finds considerable utility in solid phase immunoadsorbant assays. By using the fusion protein, it is possible to coat solid supports, such as microtitration plates, with PspA fragments without having first to isolate the PspA fragments. This may be done by adding bacterial extract containing the fusion protein to plates coated with G_{M1}. The PspA-CTB fusion protein then binds to G_{M1} through the CTB moiety, thereby coating the solid support with PspA. The resulting coated product then may be used in a solid phase immunoadsorbant assay for the detection of PspA antibody and/or antigen in test samples. Such immunoadsorbant assays constitute an additional aspect of this invention.

The PspA attachment/anchor region, containing the proline-rich region, the repeat region and/or the C-terminus of PspA, also may be employed to effect expression of heterologous proteins in pneumococci, or other gram positive or gram negative bacteria in the which the attachment/anchor region is functional. Generally, expression is effected on bacterial membrane, cell walls or cell surfaces in gram positive bacteria and in the periplasm of gram negative bacteria. An example of such heterologous protein is the B-subunit of cholera toxin.

As mentioned above, the truncated form of PspA provided herein contains the immunoprotective epitopes of the protein and hence is useful in a vaccine against

pneumococcal infection. Accordingly, a yet further aspect of the present invention provides a vaccine against pneumococcal infection comprising, as an immunogenically-active component, the purified immunoprotective pneumococcal surface protein provided herein. The PspA protein may be employed as one component of a multicomponent vaccine which is effective in providing protection from a variety of infections.

In addition, gram positive bacteria which have been transformed to express the pspA gene coding for the truncated soluble PspA protein may be employed, in a live-attenuated or killed form, as an immunologically-active component of a vaccine against pneumococcal infection. In the transformed bacterium, such pspA gene may be fused to a gene coding for another protein. Accordingly, an additional aspect of this invention provides a vaccine against pneumococcal infection comprising, as an immunologically-active component, a live-attenuated or killed bacteria containing a gene coding for the truncated form of PspA.

The truncated form of PspA also may be employed in conjugates with normally weakly-immunogenic or non-immunogenic protection-eliciting molecules, such as various polysaccharides, to achieve immunogenic potentiation thereof. An additional aspect of the invention, therefore, provides a vaccine comprising, as an immunogenically-active component, a conjugate of the purified immunoprotective pneumococcal surface protein provided herein and a normally weakly-immunogenic or non-immunogenic protection-eliciting molecule.

Conserved sequences of pspA, particularly those in the proline-rich and/or repeat regions of the gene, may be employed as probes to detect the presence of pneumococci of various strains, through detection of pneumococcal DNA, in tissues, body fluids and/or

secretions. Similarly, portions of the pspA gene may be used in diagnostic kits for the detection of pneumococcal infections.

In addition, primers made based on conserved sequences of pspA, particularly those in the proline-rich and/or repeat regions, may be used to assay for the presence of pneumococci in tissues, body fluids and/or secretions, through amplification of pneumococcal DNA. In this regard, a single primer pair derived from the nucleotide sequence of the pspA gene of S.pneumoniae may be employed in an assay using the polymerase chain reaction (PCR) for the specific detection of Streptococcus pneumoniae.

Specific amplification has been achieved of a 678 base pair DNA fragment from S.pneumoniae strain Rx1. After 30 cycles of amplification, the amplimer was detectable by agarose gel electrophoresis. The fragment was successfully amplified in all 32 strains of S.pneumoniae tested. PCR DNA amplification was able to detect less than an estimated 20 ficograms total genomic pneumococcal DNA.

Primers LSM1 and LSM2, having the nucleotide sequences:

LSM1 5'-CCGGATCCAGCTCCTGCACCAAAAC-3'

LSM2 5'-GCGCTGCGACGGCTTAAACCCATTCACCATTGG-3'

amplified the 678 base pair product from pspA from nucleotides 1312 to 1990 of the Rx1 pspA sequence (Figure 3).

The PCR analysis using the primers described herein is performed in accordance with conventional PCR techniques, such as are described in the literature, for example, as described in Arnhem et al at C&EN Special Report, 36, October 1, 1990. For detection purposes, the primer may be labelled or labelled nucleotide triphosphates may be included in the PCR reaction to label the PCR amplification product.

The PCR primers may be prepared by well-known methods, for example, by oligonucleotide synthesis or by fragmentation of a larger nucleotide sequence using suitable restriction enzymes.

The ability to use a single primer capable of detecting a large number of S.pneumoniae strains enables a universal PCR detection kit to be provided which is able to diagnose pneumococcal infection in mammals, including humans, independent of the strain which has caused the disease.

STRAINS, PLASMIDS AND PROBES

In the Examples which follow as well as in the accompanying drawings, reference is made to certain plasmids and bacterial strains transformed by such plasmids as well as vector DNA segments, some of which have been deposited with ATCC and all of which are fully described herein. The following Table II provides a summary of such materials.

TABLE II

Identification	Type	Description	Deposit	Location
JY4313	*E.coli* strain	PspA DNA	ATCC 68529	Fig. 1
JY2008	*S.pneumomiae* strain	PspA fragment 43 kDa	ATCC 55143	Fig. 1
JY4306	*E.coli* strain	PspA fragment 43 kDa	ATCC 68522	Fig. 3
JY4310		PspA fragment 21 kDa	None	Fig. 7
JY4285		PspA fragment 18 kDa	None	Fig. 7
pJY4163	Plasmid	Expression plasmid used for expression of PspA -CTB fusion protein (29 kDa)	None	Fig. 6
JY4323	DNA probe	*Hin*dIII-*Kpa*I segment	None	Fig. 9
JY4306	DNA probe	*Hin*dIII-*Dra*-I segment	None	Fig. 9
JY4262	DNA probe	*Bcl*I-*Bst*-NI segment	None	Fig. 9

EXAMPLES

Example 1

This Example illustrates the preparation and growth of novel strains of *S. pneumoniae*.

The *S. pneumoniae* strain Rx1, which is a non-encapsulated derivative of capsular type 2 strain D39 (National Collection of Type Cultures, London, NCTC #7466), was subjected to insertional inactivation (as described in McDaniel et al (III) 1987, Crain et al 1990, Talkington et al 1991, with 10 different cloned fragments of PspA (see Figure 4). These fragments have all been obtained from restriction digests of cloned PspA DNA on a plasmid in *E.coli* strain JY4313 (deposited with the American Type Culture Collection on January 31, 1991 under ATCC accession number 68529). This insertional duplication mutagenesis (see Figure 4) results in the termination of gene expression near the 3′ end of the cloned fragment.

One of the resultant strains, JY2008 (deposited with the American Type Culture Collection on January 24, 1991 under accession number 55143), which was produced by a fragment of DNA encoded in pKSD300 (McDaniel et al (III) 1987), produces a PspA fragment of 27 kDa (apparent molecular weight 43 kDa). This fragment is approximately 40% the size of the native 65 kDa (84 kDa apparent size) protein.

The expected molecular size is based on the deduced amino acid sequence and the apparent molecular size is based on migration in SDS-PAGE. The difference between expected and apparent molecular size is due to the conformation of the PspA fragment.

The proline and repeats/anchor regions (see Figure 1) were deleted and the resulting protein was unable to attach to cell due to their absence. The unattached protein then may be isolated from culture supernatants, as described below.

By directing the insertion to different points in the *pspA* gene, different lengths of truncated, non-

attached PspA protein derivatives can be produced, as seen in Figure 7.

The pneumococcal strain JY2008 was grown in 6 liters of a chemically defined medium (see Inf. Imm. 27:444) supplemented with 0.10% choline chloride, 0.075% L-cysteine hydrochloride and 0.25% $NaHCO_3$. The supernatant fluid of the mid-log phase culture of JY2008 was harvested using a 0.22 μm membrane tangential flow filter and concentrated 60 fold.

Introduction of the plasmid pKSD300 into the unmodified D39 strain similarly yielded the 43 kD truncated PspA protein. Introduction of the plasmid pKSD300 into the type 3 S.pneumoniae strain WU2 (PspA protein approximately 92 kD) yielded, upon growth of the organism, a non-attached truncated PspA protein of approximately 46 kD molecule size.

Example 2

This Example illustrates the purification of PspA.

The concentrated supernatant fluid, produced as described in Example 1, was washed in 0.1 M PBS, pH 7.2, and ultracentrifuged at 196,000 xg. The supernatant fluid was diluted 1:5 in 20 mM L-histidine buffer-NaCl, the pH adjusted to 6.0 and the injected into a DEAE-fibered Isonet-D2 an ion exchange column.

A stepwise NaCl gradient from 80 mM to 2 M was applied to the column and PspA-containing fractions (0.32 to 0.64 M ionic strength) were pooled and separated on an SDA-polyacrylamide gel. The proteins on a representative section of the gel were stained with Comassie Blue R-250 to identify PspA. The fraction containing PspA was excised from the remainder of the SDS-gel and electroeluted from the excised gel. The eluted protein was precipitated in a 50:50 methanol:acetone solvent and resuspended in PBS. Purity of product was confirmed by silver staining and Western Immunoblotting with mAb Xi126 (IgG 2b, k, see McDaniel et al (I), supra).

Example 3

This Example illustrates the isolation of PspA from the periplasmic space of Escherichia coli.

Isolation from the periplasmic space of E. coli was accomplished by standard techniques. E. coli strain JY4306 (which produces the 43 kDa N-terminal fragment of PspA, the amino acid sequence of which is shown in Figure 3. This strain was deposited with ATCC on January 31, 1991 under accession number 68522) was washed in buffered saline, incubated in 20% sucrose, 10mM EDTA, 25mM Tris pH 7.7 for 10 minutes at 0°C. The cells then were spun at 400 xg for 10 minutes at 0°C. All supernatant was removed from the pellet and the pellet was resuspended rapidly in about 100 volumes of 4°C water. After 10 minutes the suspension was centrifuged at 4,000 xg for 10 minutes at 4°C. The pellet was discarded and the supernatant, which contained the PspA was saved. Concentration of the supernatant was by standard procedures such as concentration against solid sucrose or ultrafiltration. Purification of the protein isolated from E. coli proceeded by the same chromatography techniques used for the isolated of the 43 kDa (truncated) PspA from the media of growing pneumococci.

Example 4

This Example illustrates the immunogenic properties of the PspA protein.

Sixteen 7-week old CBA/N mice carrying the Xid mutation (Jackson Laboratories, Bar Harber, ME) were bled via the periorbital sinus to establish pre-exposure levels of antibody to PspA. Purified PspA, prepared as described in Example 2, was emulsified in complete Freund's adjuvant and injected subcutaneously into the inguinal and axillary regions, delivering approximately 5 μg of protein per mouse. Fourteen days later, the mice were injected intraperitoneally with 5 μg of PspA,

prepared as described in Example 2. Control mice were immunized via the same routes with sterile SDS buffer. Seven days after the last immunization, all mice were bled via the periorbital sinus and were challenged intravenously with 300 CFU of the type 3 strain WU2, grown as described in Example 1.

Preimmunization and prechallenge sera were analyzed by Western immunoblots to establish baseline and postimmunization response to the truncated protein. The PspA of strain WU2 was electrophoresed and transferred to nitrocellulose membranes. The membranes were separated into strips and probed with the appropriate mouse antisera at a 1:50 dilution for 2 hours, incubated with biotinylated goat anti-mouse immunoglobulin for 1 hr, washed and incubated with Strepavidin-conjugated phosphatase. The membranes were developed with 5-bromo-4-chloro-3-indoyl phosphate toludine salt with 0.01% into blue tetrazolium.

Of the eight CBA/N mice immunized with the purified fragment of PspA, all were still alive 14 days after challenge with strain WU2 and none showed any signs of illness following challenge. Of the eight mice immunized with buffer controls, six were dead by two days post challenge, while the two remaining control mice appeared very sick, with ruffled fur, arched back and decreased movement, two to three days following challenge but survived. Chi-square analysis indicated that there was a significant difference ($P < 0.003$) in survival between the immunized and control groups.

Preimmunization and prechallenge sera were analyzed by Western immunoblotting. None of the preimmunization sera contained antibody to truncated PspA. Postimmunization sera from eight of eight mice contained detectable antibodies to PspA, and six mice had very strong anti-PspA reactions. When the challenge strain WU2 was probed with the antisera, all the immunized mice

had antibodies that were highly cross-reactive with the WU2 PspA epitopes. No control mice developed antibodies to PspA.

The immunization data is summarized in the following Table III:

Table III

Immunogen	Detection of Antibody to PspA	Alive at 2 days post challenge	Alive at 14 days post challenge
Isolated PspA (Example 2)	8/8	8/8	8/8
Sterile SDS (control)	0/8	2/8	2/8

As may be seen from the data in Table III, immunization with two 5 μg doses of the purified PspA molecule elicited protection against fatal infection of CBA/N mice and elicited antibodies reactive with the PspA of the challenge strain.

Example 5

This Example illustrates sequencing of the PspA protein.

Purified PspA, prepared as described in Example 2, was electrophoresed through 9% resolving gels containing recrystallized SDS with the Laemmli buffer system (Nature 227:680). The gels were soaked twice in a 10mM 3-(cyclohexylamino)-1-propanesulfonic acid, pH 11.0, containing 10% methanol for 10 minutes. A polyvinylidene difluoride membrane (PVDF) was wetted completely for several seconds in 100% methanol, then washed in CAPS buffer for 10 min. PspA was electrotransferred to the PVDF membrane in CAPS buffer at 0.5 A for 1 hr. After transfer, the membrane was washed two times in deionized water for 5 min, and stained with 0.1% Coomassie Blue R-250 in 50% methanol for 20 minutes. The section of the membrane containing PspA was excised and destained in 40% methanol and 10% acetic acid for 5 min. The membrane was cut into small

segments and stored in sterile Eppendorf tubes until sequencing.

The isolated PspA was sequenced directly from the PVDF membranes. Figure 2 depicts the N-terminal 45 residue amino acid sequence and Figure 5 depicts the amino acid sequence for the whole alpha-helical region. The DNA sequence of the whole pspA gene and the deduced amino acid sequence for the PspA protein are shown in Figure 3.

Example 6

This Example illustrates the use of the pspA 5'-sequence and/or the PspA N-terminal region to serve as an expression and leader sequence for expressing and/or excreting/secreting heterologous proteins from S.pneumoniae and E.coli. In this Example, there is described the expression of the N-terminal of the PspA protein fused to the B-subunit of cholera toxin (CTB) through a genetic fusion and the excretion of the fused protein from pneumococci and its secretion into the periplasmic space of E.coli.

A fusion protein consisting of CTB and the N-terminal half of PspA was constructed and expressed in E.coli. The HindIII/DraI pspA gene fragment used contained all the pspA transcription and translation initiation signals and the PspA signal peptide leader sequence for transport across the cell membrane. The mature PspA encoded by this fragment is predicted to be a product of 29 kDa (observed molecular weight of 42 kDa), encompassing more than 90% of the α-helical coiled-coil domain. The CTB fragment used lacked transcription and translation initiation signals. Expression from pspA promoter through pspA and then in-frame translational readthrough into the CTB-encoding gene ctxB resulted in production of a 12 kDa CTB product fused to the upstream PspA product. The PspA-CTB fusion protein was stably expressed in both high and low copy

number plasmids (pUC18, more than 100 copies/cell; pJY4163, about 15 to 30 copies/cell) in E.coli.

The fusion products were of the expected size (about 54 kDa) and reacted with antibody to both PspA and CTB. That the CTB product retained its functionality was demonstrated by the ability of the fusion protein to bind ganglioside G_{M1}, a property of CTB.

The high level of expression of the fusion product apparently resulted in a reduced rate of processing and/or conformational changes that prevented the protein from being completely transported to the periplasm. However, in the lower copy number construct, about 60% of the fusion protein was localized in the periplasm, where large quantities were readily released from E. coli by osmotic shock.

In addition to expression in E.coli, the fusion protein also was expressed in S.pneumoniae by transformation of the low copy number construct into the avirulent S.pneumoniae Rx1 to generate an insertion-duplication mutant. In this way, the gene encoding the fusion protein was integrated into the S.pneumoniae chromosome, from which it was stably expressed. As in the case of Example 1, the truncated PspA molecule lacking the attachment/anchor region, this time in the form of the PspA-CTB fusion protein, was excreted into the culture supernatant. The fusion protein product was of the expected molecular size (54 kDa), reacted with antibody to PspA and CTB, and bound G_{M1}.

Example 7

This Example illustrates the use of PspA attachment or anchor region to permit expression of heterologous proteins on the surface of S.pneumoniae or other bacteria in which the attachment/anchor sequence is functional in particular the expression of a PspA-CTB

(cholera toxin B subunit) fusion expressed on the surface of pneumococci.

The N-terminal encoding region of PspA, including its transcription and translation initiation signals and its signal peptide leader sequence, is linked via a translationally in-frame genetic fusion to the CTB-encoding ctxB fragment that lacks transcription and translation initiation and termination signals. This sequence is followed in-frame by the PspA attachment/anchor domain, including part or all of the proline, repeat and C-terminal domains. The resulting fusion protein is directed to the outside of the cell via the PspA leader sequence, which is cleaned following transport across the membrane, and then attached to cell by the PspA attachment/anchor sequence. The heterologous protein, located between the two PspA fragments is expressed on the outside surface of the membrane and, in S.pneumoniae, on the surface of the cell.

Example 8

This Example illustrates the expression of truncated and full length PspA by the Mycobacterium tuberculosis strain Bacille Calmette-Guerin (BCG).

BCG was chromosomically modified to incorporate the pspA gene coding for the truncated PspA protein. The 43 kDa truncated PspA protein was expressed from the modified BCG to approximately 15% of total BCG protein. This result was achieved with an expression vector construct carrying the pspA gene segment encoding the 43 kDa region without its 5′-secretion signal. Expression was only about 1% of BCG protein when the PspA or mycobacterial signal sequences were included. In either case, a significant portion of the expressed PspA was excreted into the medium. Expression of the 43 kDa PspA protein in a fusion with the mycobacterial lipoprotein

signal sequence resulted in the expression of the recombinant PspA in the membrane fraction of BCG.

This latter result suggested that the fusion of the lipoprotein signal sequence resulted in acylation of the recombinant PspA. Fluorescent activated cell sorting with fluorochrome-conjugated monoclonal antibodies to PspA demonstrated expression of PspA on the surface of these bacteria.

Example 9

This Example illustrates the close homology of portions of the *pspA* gene among different strains of pneumococci and the potential that such homology may be useful for molecular (DNA) approaches to detect pneumococci in tissues, body fluids and/or secretions and to identify pneumococci grown from patient samples.

Three DNA probes were employed, namely full length *pspA*, JY4323 (N-terminal *Hin*dIII to C-terminal *Kpn*I), the N-terminal half of *pspA*, JY4306 (N-terminal *Hin*dIII to *Dra*I at position 996 (see Figure 3) and most of the proline and repeat regions, JY4262 (*Bcl*I at position 1221 to *Bst*NI at position 1832). Under stringency conditions requiring 95% identity, probes JY4323 and JY4262 reacted with cells of over 200 independent isolates of *S.pneumoniae* by Southern blot.

When the chromosomal DNA was cut with *Hin*dIII, there generally was observed that each of these probes detected two discrete bands whose exact size was dependent on the strain examined. In Rx1 pneumococci, the two bands were 4.0 and 9.1 kb. The 4.0 kb band corresponded to *pspA* and was altered or absent in *pspA* mutants. The other band shares some homology with the coding regions for both the N-terminal and C-terminal halves of PspA but is not affected by *pspA* mutations. The JY4323 and JY4262 probes failed to react with another gram positive bacterium, *Streptococcus pyogenes*, and a gram negative bacterium, *Salmonella typhimurium*.

The N-terminal probe, JY4306, recognized about one-third of the strains of pneumococci tested.

These results indicate that a sequence included in the proline/repeat region is shared by all strains of pneumococci and apparently not by other bacterial species. Sequences in the N-terminal half of the molecule appear to be more variable.

Example 10

This Example illustrates the detection and determination of the location of epitopes in the α-helical N-terminal region of PspA.

Monoclonal antibodies protective against pneumococcal infection in a mouse model, denoted by an asterisk in Figures 5, 6 and 7, were used to determine the location of epitopes for each antibody in the α-helical N-terminal region of PspA. The sites were mapped to fragments of PspA. The results are illustrated in Figures 5 to 7, with Figure 5 showing the deduced amino acid sequence for the N-terminal region of PspA and the general location of epitopes recognized by monoclonal antibodies, Figure 6 showing antibody reactivity with PspA fragments produced by various pspA gene segments, and Figure 7 showing the mapped location of epitopes in the PspA fragments produced by the different pspA gene segments.

Numbers 138, 193 and 261 in Figure 5 indicate break positions in the PspA fragments used to map the location of epitopes detected by monoclonal antibodies Xi1526, Xi126, XiR35, XiR38, XiR1224, XiR16, Xi64, XiR278, Xi1325 and Xi1323. The asterisk (*) after some of the antibodies denotes those which are able to protect against fatal pneumococcal infection with strain WU2 pneumococci.

In addition, the vertical lines to the right of the Figure indicate those areas predicted to have coiled-coil α-helical structure. The divisions to the left of

the Figure indicate the mapped location of the epitopes for each antibody.

SUMMARY OF DISCLOSURE

In summary of this disclosure, the present invention relates to a truncated PspA molecule capable of eliciting an immunoprotective response and hence containing the protective epitopes of PspA protein. Modifications are possible within the scope of this invention.

CLAIMS

What we claim is:

1. A purified immunoprotective pneumococcal surface protein (PspA), comprising a truncated form of PspA which contains the immunoprotective epitopes of the protein and up to about 90% of the whole PspA protein and from which the functional cell membrane anchor region is absent.

2. The protein of claim 1 containing approximately 50% of the whole PspA protein from which the cell membrane anchor region, the repeat region and the proline region are absent.

3. The protein of claim 1 comprising at least the N-terminal, protective epitope-containing region of the PspA protein.

4. The protein of claim 1 comprising at least the N-terminal, protective epitope-containing region of the PspA protein having the domains shown in Figure 1.

5. The protein of claim 1 comprising an N-terminal α-helical coil region of the whole PspA protein.

6. The protein of claim 5 wherein said α-helical coil region has a seven residue periodicity.

7. The protein of claim 1 comprising the 43 kD (apparent molecular size) N-terminal region of an 84 kD PspA protein.

8. A biologically-pure recombinant DNA molecule coding for at least a portion of the PspA protein and having a coding sequence included in that set forth in Figure 3 or having substantial homology thereto.

9. The DNA molecule of claim 8 coding for at least part of an α-helical coiled coil region of the PspA protein.

10. The DNA molecule of claim 8 coding for at least part of a proline rich region of the PspA protein.

11. The DNA molecule of claim 8 coding for at least part of a repeat region of the PspA protein.

12. A biologically-pure recombinant DNA molecule coding for a truncated form of the PspA protein which contains the immunoprotective epitopes of the protein and contains up to about 90% of the whole PspA protein from which the cell membrane anchor region is absent, said DNA molecule having a coding sequence included in that set forth in Figure 3 or having substantial homology thereto.

13. The DNA molecule of claim 12 which codes for a truncated form of the PspA protein which contains approximately 50% of the whole PspA protein from which the cell membrane anchor region, the repeats region and the proline region are absent.

14. The DNA molecule of claim 12 which is linked by a translationally in-frame genetic fusion to a gene coding for another protein.

15. The DNA molecule of claim 14 wherein said gene coding for another protein is a ctxB gene.

16. A method of forming an immunoprotective truncated PspA protein, which comprises:

effecting insertion-duplication mutagenesis of a bacterium with a pspA gene resulting in the coding of a truncated expressible PspA protein,

growing said mutated bacterium to effect expression of a truncated PspA protein, and

isolating said protein.

17. The method of claim 16 wherein said bacterium comprises an S.pneumoniae strain, said mutated strain is grown in a defined medium to effect expression of the truncated PspA protein into said medium, and the protein is isolated from the medium.

18. The method of claim 17 wherein said mutated strain is S.pneumoniae strain JY2008.

19. The method of claim 16 wherein said bacterium comprises an E.coli strain, said mutated strain is grown in a defined medium to effect expression of the

truncated PspA protein into the periplasm of said mutated strain, and the truncated protein is isolated from the periplasm of said mutated strain.

20. The method of claim 19 wherein said mutated strain is E.coli strain JY4306.

21. The method of claim 16 wherein said bacterium comprises a Mycobacteria strain.

22. The method of claim 21 wherein said mutated strain is mutated BCG.

23. The method of claim 16 wherein said isolated protein is purified by ion-exchange chromatography.

24. A mutated strain of Streptococcus pneumoniae comprising a pspA gene coding for a truncated expressible PspA protein.

25. The strain of claim 24 which is the JY2008 strain of S.pneumoniae.

26. The strain of claim 24 which is the JY4310 strain of S.pneumoniae.

27. The strain of claim 24 which is the JY4285 strain of S.pneumoniae.

28. The strain of claim 24 possessing a pspA gene coding for a truncated PspA protein linked to a gene coding for another protein.

29. The strain of claim 28 wherein said gene coding for another protein is the ctxB gene coding for the B-subunit of cholera toxin.

30. The strain of claim 29 which is the JY2182 strain of S.pneumoniae.

31. A mutated strain of Mycobacterium comprising a pspA gene coding for a truncated expressible PspA protein.

32. The strain of claim 31 which is mutated BCG.

33. A mutated strain of Eschericia coli possessing a pspA gene coding for a truncated expressible PspA protein.

34. The strain of claim 33 which is the JY4306 strain of E. coli.

35. The strain of claim 33 which is the JY4353 strain of E.coli.

36. A method for the production of cloned proteins which comprises:

fusing a pspA gene coding for a truncated form of PspA protein with the gene coding for another protein to form a fusion protein clone,

transforming a bacterium with said fusion protein clone,

growing the transformed bacterium to effect expression of a fusion protein comprising truncated PspA and said other protein, and

isolating said fusion protein.

37. The method of claim 36 wherein said bacterium is a gram positive bacterium, the transformed gram positive bacterium is grown in a defined medium to effect expression of the fusion protein into said medium, and the fusion protein is isolated from the medium.

38. The method of claim 37 wherein said gram positive bacterium is a strain of S. pneumoniae.

39. The method of claim 37 wherein said gram positive bacterium is a strain of Mycobacterium.

40. The method of claim 36 wherein said bacterium is a gram negative bacterium, the transformed gram negative bacterium is grown in a defined medium to effect expression of the fusion protein into the periplasm of said bacterium, and the fusion protein is isolated from said periplasm.

41. The method of claim 40 wherein said gram negative bacterium is a strain of E. coli.

42. The method of claim 36 wherein said gene coding for another protein is a gene coding for the B-subunit of cholera toxin (CTB).

43. The method of claim 42 wherein said fusion protein comprises an acid labile sequence joining said truncated form of PspA protein and CTB and said PspA protein and CTB are separated from each other by dilute acid treatment.

44. The method of claim 42 wherein said fusion protein is isolated from culture medium by adsorption to a G_{M1} affinity column, from which said fusion protein is eluted.

45. A vaccine against pneumococcal infection, comprising, as an immunogenically-active component, the protein defined in claim 1.

46. The vaccine of claim 45 wherein said protein is conjugated to a normally weakly-immunogenic or non-immunogenic protection-eliciting molecule.

47. A vaccine against pneumococcal infection, comprising, as an immunologically activated component, a live-attenuated or killed gram positive bacteria containing a gene coding for the protein defined in claim 1.

48. The vaccine of claim 47 wherein said gram positive bacteria is <u>Streptococcus pneumoniae</u>.

49. The vaccine of claim 47 wherein said gram positive bacteria is <u>Mycobacterium tuberculosis</u>.

50. A DNA probe for the detection of pneumococcal DNA in a sample, comprising a conserved sequence of the <u>pspA</u> gene.

51. The DNA probe of claim 50 wherein said conserved sequence is located in the proline-rich region of the gene, the repeat region of the gene or both.

52. The DNA probe JY4262.

53. The DNA probe JY4323.

54. A DNA primer for effecting polymerase chain reaction of pneumococcal DNA comprising a conserved sequence of the <u>pspA</u> gene for assaying a sample for the presence of pneumococci.

55. The DNA primer of claim 54 wherein said conserved sequence is located in the proline-rich region of the gene, the repeat region of the gene or both.

56. The DNA primer of claim 55 wherein said conserved sequence comprises a 678 base pair fragment of S. pneumoniae strain Rx1 extending from nucleotide 1312 to 1990 of the pspA sequence of Figure 3.

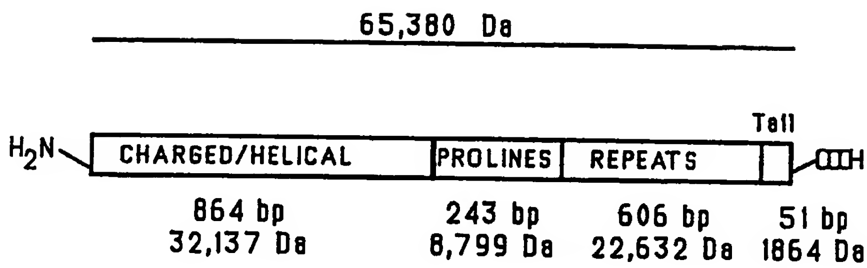
57. The DNA primer of claim 55 wherein said primer has the nucleotide sequence:

5'-CCGGATCCAGCTCCTGCACCAAAAC-3' (LSM1)

or

5'-GCGCTGCGACGGCTTAAACCCATTCACCATTGG-3' (LSM2)

58. In a solid phase immunoabsorbant assay, the improvement which comprises a coating of a truncated form of PspA protein on a solid substrate wherein said PspA protein is fused to the B-subunit of cholera toxin (CTB) which is bound to monosinloganglioside (G_{M1}) coating said solid substrate.



DOMAINS OF THE MATURE PspA

FIG.1.

							a	b	c	d	e	f	g	
GLU	GLU	ser	pro	val	ala	ser	gln	ser	LYS	ala	GLU	LYS	ASP	14
							tyr	ASP	ala	ala	LYS	LYS	ASP	21
							ala	LYS	asn	ala	LYS	LYS	ala	28
							val	GLU	ASP	ala	gln	LYS	ala	35
							leu	ASP	ASP	ala	LYS	ala	ala	42
							gln	LYS	LYS					45

FIG.2.

2/8

FIG.3a.

```
pspa - sequence -> 1-phase Translation
DNA and derived amino acid 2086 b.p. AAGCTTATGATA.....TCTTTAGGTACC linear
1   /   1                                       31  /   11
AAG CCT ATG ATA TAG AAA TTT GTA ACA AAA ATG TAA TAT AAA ACA CTT GAC AAA TAT TTA

61  /   21                                      91  /   31
CGG AGG AGG CTT ATA CTT AAT ATA AGT ATA GTC TGA AAA TGA CTA TCA GAA AAG AGG TAA

121 /   41                                      151 /   51
ATT TAG ATG AAT AAG AAA AAA ATG ATT TTA ACA AGT CTA GCC AGC GTC GCT ATC TTA GGG
        met asn lys lys lys met ile leu thr ser leu ala ser val ala ile leu gly
181 /   61                                      211 /   71
GCT GGT TTT GTT GCG TCT CAG CCT ACT GTT GTA AGA GCA GAA GAA TCT CCC GTA GCC AGT
ala gly phe val ala ser gln pro thr val val arg ala glu glu ser pro val ala ser
241 /   81                                      271 /   91
CAG TCT AAA GCT GAG AAA GAC TAT GAT GCA GCG AAG AAA GAT GCT AAG AAT GCG AAA AAA
gln ser lys ala glu lys asp tyr asp ala ala lys lys asp ala lys asn ala lys lys
301 /   101                                     331 /   111
GCA GTA GAA GAT GCT CAA AAG GCT TTA GAT GAT GCA AAA GCT GCT CAG AAA AAA TAT GAC
ala val glu asp ala gln lys ala leu asp asp ala lys ala ala gln lys lys tyr asp
361 /   121                                     391 /   131
GAG GAT CAG AAG AAA ACT GAG GAG AAA GCC GCG CTA GAA AAA GCA GCG TCT GAA GAG ATG
glu asp gln lys lys thr glu glu lys ala ala leu glu lys ala ala ser glu glu met
421 /   141                                     451 /   151
GAT AAG GCA GTG GCA GCA GTT CAA CAA GCG TAT CTA GCC TAT CAA CAA GCT ACA CAC AAA
asp lys ala val ala ala val gln gln ala tyr leu ala tyr gln gln ala thr asp lys
481 /   161                                     511 /   171
GCC GCA AAA GAC GCA GCA GAT AAG ATG ATA GAT GAA GCT AAG AAA CGC GAA GAA GAG GCA
ala ala lys asp ala ala asp lys met ile asp glu ala lys lys arg glu glu glu ala
541 /   181                                     571 /   191
AAA ACT AAA TTT AAT ACT GTT CGA GCA ATG GTA GTT CCT GAG CCA GAG CAG TTG GCT GAG
lys thr lys phe asn thr val arg ala met val val pro glu pro glu gln leu ala glu
601 /   201                                     631 /   211
ACT AAG AAA AAA TCA GAA GAA GCT AAA CAA AAA GCA CCA GAA CTT ACT AAA AAA CTA GAA
thr lys lys lys ser glu glu ala lys gln lys ala pro glu leu thr lys lys leu glu
```

FIG.3b.

```
661     /   221                                         691     /   231
GAA GCT AAA GCA AAA TTA GAA GAG GCT GAG AAA AAA GCT ACT GAA GCC AAA CAA AAA GTG
glu ala lys ala lys leu glu glu ala glu lys lys ala thr glu ala lys gln lys val
721     /   241                                         751     /   251
GAT GCT GAA GAA GTC GCT CCT CAA GCT AAA ATC GCT GAA TTG GAA AAT CAA GTT CAT AGA
asp ala glu glu val ala pro gln ala lys ile ala glu leu glu asn gln val his arg
781     /   261                                         811     /   271
CTA GAA CAA GAG CTC AAA GAG ATT GAT GAG TCT GAA TCA GAA CAT TAT GCT AAA GAA GGT
leu glu gln glu leu lys glu ile asp glu ser glu ser glu asp tyr ala lys glu gly
841     /   281                                         871     /   291
TTC CCT GCT CCT CTT CAA TCT AAA TTG GAT GCG AAA AAA GCT AAA CTA TCA AAA CTT GAA
phe arg ala pro leu gln ser lys leu asp ala lys lys ala lys leu ser lys leu glu
901     /   301                                         931     /   311
CAG TTA AGT GAT AAG ATT GAT GAG TTA GAC GCT GAA ATT GCA AAA CTT GAA GAT CAA CTT
glu leu ser asp lys ile asp glu leu asp ala glu ile ala lys leu glu asp gln leu
961     /   321                                         991     /   331
AAA GCT GCT GAA GAA AAC AAT AAT GTA GAA GAC TAC TTT AAA GAA GGT TTA GAG AAA ACT
lys ala ala glu glu asn asn asn val glu asp tyr phe lys glu gly leu glu lys thr
1021    /   341                                         1051    /   351
ATT GCT GCT AAA AAA GCT GAA TTA GAA AAA ACT GAA GCT GAC CTT AAG AAA GCA GTT AAT
ile ala ala lys lys ala glu leu glu lys thr glu ala asp leu lys lys ala val asn
1081    /   361                                         1111    /   371
GAG CCA GAA AAA CCA GCT CCA GCT CCA GAA ACT CCA GCC CCA GAA CCA CCA GCT GAA CAA
glu pro glu lys pro ala pro ala pro glu thr pro ala pro glu ala pro ala glu gln
1141    /   381                                         1171    /   391
CCA AAA CCA GCG CCG GCT CCT CAA CCA GCT CCC GCA CCA AAA CCA GAG AAG CCA GCT GAA
pro lys pro ala pro ala pro gln pro ala pro ala pro lys pro glu lys pro ala glu
1201    /   401                                         1231    /   411
CAA CCA AAA CCA CAA AAA ACA GAT GAT CAA CAA CCT GAA GAA GAC TAT GCT CGT AGA TCA
gln pro lys pro glu lys thr asp asp gln gln ala GLU glu asp tyr ala arg arg ser
1261    /   421                                         1291    /   431
GAA GAA GAA TAT AAT GGC TTG ACT CAA CAG CAA CCG CCA AAA CTT CAA AAA CCA GCT CCT
glu glu glu tyr asn arg leu thr gln gln gln pro pro lys ala glu lys pro ala pro
1321    /   441                                         1351    /   451
GCA CCA CCA ACA GGC TGG AAA CAA GAA AAC GGT ATG TGG TAC TTC TAC AAT ACT GAT GGT
ala pro lys thr gly trp lys gln glu asn gly met trp tyr phe tyr asn thr asp gly
```

FIG.3c

1381 / 461 1411 / 471
TCA ATG GCG ACA GGA TGG CTC CAA AAC AAC GGT TCA TGG TAC TAC CTC AAC AGC AAT GGT
ser met ala thr gly trp leu gln asn asn gly ser trp tyr tyr leu asn ser asn gly
1441 / 481 1471 / 491
GCT ATG GCT ACA GGT TGG CTC CAA TAC AAT GGT TCA TGG TAT TAC CTC AAC GCT AAC GGC
ala met ala thr gly trp leu gln tyr asn gly ser trp tyr tyr leu asn ala asn gly
1501 / 501 1531 / 511
GCT ATG GCA ACA GGT TGG GCT AAA GTC AAC GGT TCA TGG TAC TAC CTC AAC GCT AAT GGT
ala met ala thr gly trp ala lys val asn gly ser trp tyr tyr leu asn ala asn gly
1561 / 521 1591 / 531
GCT ATG GCT ACA GGT TGG CTC CAA TAC AAC GGT TCA TGG TAT TAC CTC AAC GCT AAC GGC
ala met ala thr gly trp leu gln tyr asn gly ser trp tyr tyr leu asn ala asn gly
1621 / 541 1651 / 551
GCT ATG GCA ACA GGT TGG GCT AAA GTC AAC GGT TCA TGG TAC TAC CTC AAC GCT AAT GGT
ala met ala thr gly trp ala lys val asn gly ser trp tyr tyr leu asn ala asn gly
1681 / 561 1711 / 571
GCT ATG GCT ACA GGT TGG CTC CAA TAC AAC GGT TCA TGG TAC TAC CTC AAC GCT AAC GGT
ala met ala thr gly trp leu gln tyr asn gly ser trp tyr tyr leu asn ala asn gly
1741 / 581 1771 / 591
GCT ATG GCT ACA GGT TGG GCT AAA GTC AAC GGT TCA TGG TAC TAC CTC AAC GCT AAT GGT
ala met ala thr gly trp ala lys val asn gly ser trp tyr tyr leu asn ala asn gly
1801 / 601 1831 / 611
GCT ATG GCA ACA GGT TGG GTG AAA GAT GGA GAT ACC TGG TAC TAT CTT GAA GCA TCA GGT
ala met ala thr gly trp val lys asp gly asp ser trp tyr tyr leu glu ala ser gly
1861 / 621 1891 / 631
GCT ATG AAA GCA AGC CAA TGG TTC AAA GTA TCA GAT AAA TGG TAC TAT GTC AAT GGT TTA
ala met lys ala ser gln trp phe lys val ser asp lys trp tyr tyr val asn gly leu
1921 / 641 1951 / 651
GCT GCC CTT GCA GTC AAC ACA ACT GTA GAT GGC TAT AAA GTC AAT GCC AAT GGT GAA TGG
gly ala leu ala val asn thr thr val asp gly tyr lys val asn ala asn gly glu trp
1981 / 661 2011 / 671
GTT TAA GCC GAT TAA ATT AAA GCA TGT TAA GAA CAT TTG ACA TTT TAA TTT TGA AAC AAA
val OCH ala asp OCH ile lys ala cys OCH glu his leu thr phe OCH phe OPA asn lys
2041 / 681 2071 / 691
GAT AAG CTT CGA TTG AAT AGA TTT ATG TTC GTA TTC TTT AGG TAC
asp lys val arg leu asn arg phe met phe val phe phe tyr tyr

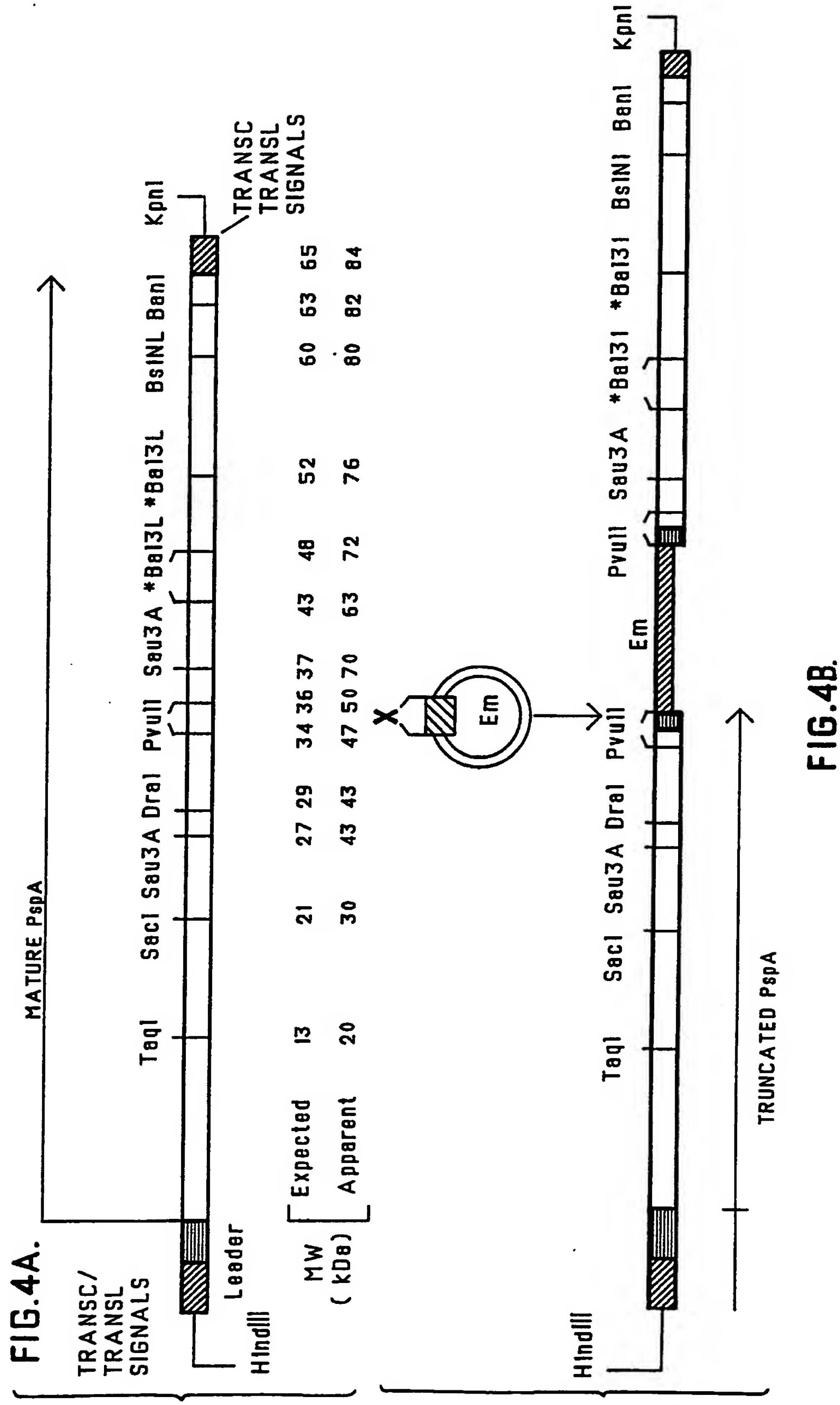

TRANSC/
TRANSL
SIGNALS
HindIII
Leader
MATURE PspA
TaqI
SacI
Sau3A
DraI
PvuII
Sau3A
*Bal3L
*Bal3L
BsINL
BanI
KpnI
TRANSC
TRANSL
SIGNALS
MW
(kDa)
Expected
13
21
27 29
34 36 37
43
48
52
60
63
65
Apparent
20
30
43 43
47 50 70
63
72
76
80
82
84
X
Em
HindIII
TaqI
SacI
Sau3A
DraI
PvuII
Em
PvuII
Sau3A
*Bal31
*Bal31
BsINI
BanI
KpnI
TRUNCATED PspA

FIG.4A.

FIG.4B.

Location of epitopes detected by monoclonal antibodies to PspA

		a	b	c	d	e	f	g
	1	E	E	s	p	v	a	s
	8	Q	s					
				K	a	E	K	D
	15	y	D	a	a	K	K	D
	22	a	K	N	a	K	K	a
	29	v	E	D	a	Q	K	a
	36	l	D	D	a	K	a	a
XI1526*	43	Q	K	K	y	D	E	D
XI126*	50	Q	K	K	t	E	E	K
XIR35	57	a	a	l	E	K	a	a
XIR148	64	s	E	E	m	D	K	a
XIR1224	71	v	a	a	v	Q	Q	a
	78	y	l	a	y	Q	Q	a
	85	t	D	K	a	a	K	D
	92	a						
					a	D	K	m
	97	l	D	E	a	K	K	R
	104	E	E	E	a	K	t	K
	111	l	N	t	v	R	a	m
	118	v	v	p	E	p	E	Q
	125	l	a	E	t	K	K	K
138 HHHHH	132	s	E	E	a	K	Q	K
	139	a	p	E	l	t	K	K
	146	l	E	E	a	K	a	K
	153	l	E	E	a	E	K	K
	160	a	t	E	a	K	Q	K
XIR16	167	v	D					
				a	E	E	v	a
	174	p	Q	a				
								K
	178	l	a	E	l	E	N	Q
	185	v	H	R	l	E	Q	E
193 HHHHH	192	l	K	E	i	D	E	s
	199	E						
					s	E	D	y
	204	a	K	E	g	l	R	a
	211	p	l	Q	s	K	l	D
	218	a	K	K	a	K	l	s
XI64*	225	K						
XIR278*								
XI1325*	226	l	E	E	l	s	D	K
	233	l	D	E	l	D	a	E
	240	l	a	K	l	E	D	Q
	247	l	K	a	a	E	E	N
	254		N	N	v	E	D	y
261 HHHHH	260	l	K	E	g	l	E	K
	267	t	l	a	a	K	K	a
	274	E						
XI1323*								
	275	l	E	K	t	E	a	D
	282	l	K	K	a	v	N	E

FIG.5.

ANTIBODY REACTIVITY

	Xi 126	XiR 1224	XiR 148	XiR 1526	XiR 35	XiR 16	XiR 278	XiR 1325	XiR 64	XiR 1323
KSD 1014	++	++	++	++	++	++	++	++	++	++
JY 4306	++	++	++	++	++	++	+	++	++	++
JY 4310	++	+	++	++	++	++	-	-	-	-
JY 4285	++	+	++	++	++	+	-	-	-	-
KSD 1500	-	-	-	-	-	-	-	-	-	-
BC 100	-	-	-	-	-	-	++	++	++	++
BC 207	-	-	-	-	-	+	++	++	++	++

FIG.6.

LOCATION OF EPITOPES

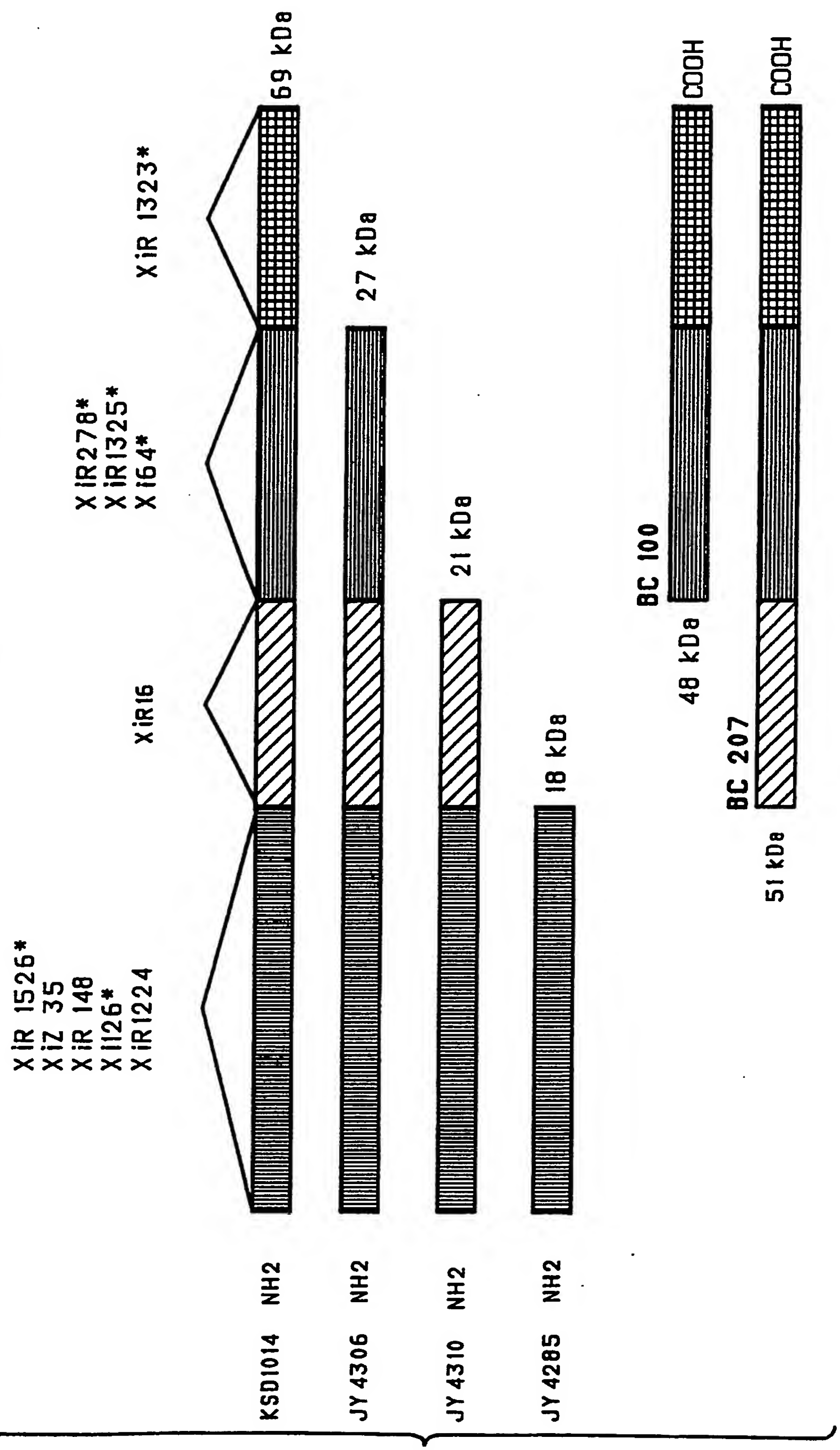


FIG.7.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/00857

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): A61K 39/12; C12N 1/20; C07H 15/12, 13/00
US CL : 530/350; 536/27; 435/253.8, 253.1, 253.4; 424/89

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
	530/350; 536/27; 435/253.8, 253.1, 253.4; 424/89

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

U.S. Automated Patent System (File USPAT, 1971-1992), Dialog (file = Biochem)

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No. [18]
Y	Abstracts of the 89th Annual Meeting of the ASM, 14-18 May 1989, McDaniel, et al., "Molecular Cloning of the Gene Encoding PspA (Pneumococcal Surface Protein A) and Analysis of Protective Immunity Induced by PspA. See Abstract No. D-255.	1-58
Y	Microbial Pathogenesis, Vol. 1, issued 1986, McDaniel, et al., "Analysis of a Surface Protein of Streptococcus pneumoniae recognized by Protective Monoclonal Antibodies", pages 519-531, see entire document.	1-58
Y	Journal of Experimental Medicine, Vol. 165 issued February 1987, McDaniel, et al., "Use of Insertional Inactivation to Facilitate Studies of Biological Properties of Pneumococcal Surface Protein A (PspA)", pages 381-394, see entire document.	1-58

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
18 MAY 1992	04 JUN 1992
International Searching Authority[1]	**Signature of Authorized Officer[20]**
ISA/US	LYNETTE SMITH

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

Please See Attached Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS

VI. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-7,16-23, 45-49 drawn to a first protein, method of making the protein and a method of using protein in a vaccine composition. Classes 530, 435, subclasses 350, 235.4 and 92.
II. Claims 8-15 drawn to a second product - DNA. Class 536, subclass 27.
III. Claims 24-35 drawn to a third product - mutant strain of Streptococcus. Class 435, subclass 253.4.
IV. Claims 36-44 drawn to a second method of producing the mutant strain of bacteria. Class 435, subclass 252.3.
V. Claims 50-57 drawn to a fourth product - DNA probes and DNA primer. Class 536, subclass 27.
VI. Claim 58 drawn to a third method of assaying the protein. Class 435, subclass 7.1.
The claims of groups I, II, III and V ar drawn to different products. The claims of group I, IV and VI are drawn to different methods of either producing the protein or using the protein. The products and methods have reached a separate status in the art as evidenced by their different classifications. PCT Rules 13.1 and 13.2 do not provide for multiple distinct methods and products within a single inventive concept.